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Maternal embryonic leucine zipper kinase (MELK) regulates multipotent neural progenitor proliferation

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Maternal embryonic leucine zipper kinase (MELK) was previously identified in a screen for genes enriched in neural progenitors. Here, we demonstrate expression of MELK by progenitors in developing and adult brain and that MELK serves as a marker for self-renewing multipotent neural progenitors (MNPs) in cultures derived from the developing forebrain and in transgenic mice. Overexpression of MELK enhances (whereas knockdown diminishes) the ability to generate neurospheres from MNPs, indicating a function in self-renewal. MELK down-regulation disrupts the production of neurogenic MNP from glial fibrillary acidic protein (GFAP)–positive progenitors in vitro. MELK expression in MNP is cell cycle regulated and inhibition of MELK expression down-regulates the expression of B-myb, which is shown to also mediate MNP proliferation. These findings indicate that MELK is necessary for proliferation of embryonic and postnatal MNP and suggest that it regulates the transition from GFAP-expressing progenitors to rapid amplifying progenitors in the postnatal brain.

Introduction

Neural stem cells are defined by their ability to self-renew, and their capacity to produce neurons, astrocytes, and oligodendrocytes (Gage, 2000; Momma et al., 2000; Panchision and McKay, 2002). In the adult subventricular zone (SVZ), slowly proliferative glial fibrillary acidic protein (GFAP)–positive cells are thought to be neural stem cells that give rise to a more rapidly proliferative, GFAP-negative progenitor (for review see Alvarez-Buylla et al., 2002). In early brain development it is not clear whether such distinctions exist, although there are large numbers of highly proliferative multipotent neural progenitors (MNP) in the periventricular neuroepithelium. MNP proliferation plays important roles in brain development, regulating cell number and brain size (Groszer et al., 2001; Molofsky et al., 2003).

Previously, we used a genome-wide screening strategy to discover genes that regulate MNP function (Geschwind et al., 2001; Easterday et al., 2003). We reasoned that at least some of the genes expressed by MNP and not by differentiated cells would be those involved in self-renewing proliferation. We used a combination of cDNA subtraction and microarray analyses to discover genes expressed in different kinds of MNP-containing neurospheres, as well as by other self-renewing populations; hematopoietic stem cells and embryonic stem (ES) cells. We then used in situ hybridization analysis to narrow this pool of genes by determining which ones were highly expressed in developing germinal zones (GZs), providing in vivo relevance to the in vitro studies (Geschwind et al., 2001; Terskikh et al., 2001; Easterday et al., 2003).

Maternal embryonic leucine zipper kinase (MELK; MPK38) (Gil et al., 1997; Heyer et al., 1997, 1999), a member of the snf1/AMPK family of serine–threonine kinases, was enriched in multiple MNP-containing populations and in hematopoietic stem cells (Easterday et al., 2003). Although several members of the family are known to play roles in cell survival under metabolically challenging conditions, the function of MELK has not previously been determined (Kato et al., 2002; Inoki et al., 2003; Suzuki et al., 2003a,b).

Here, we show that MELK is expressed by MNP derived from several ages, and is necessary for their proliferation in vitro, influencing their ability to form neurospheres, a measure

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Abbreviations used in this paper: CNS, central nervous system; EGL, external granule cell layer; ES, embryonic stem; GFAP, glial fibrillary acidic protein; GZ, germinal zone; MELK, maternal embryonic leucine zipper kinase; MNP, multipotent neuroprogenitor; Msi1, musashi1; NCS, nucleostemin; NS, neural stem; PCMV, cytomegalovirus promoter; PMELK, MELK promoter; siRNA, small inhibitory RNA; SVZ, subventricular zone.

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of self-renewal. The data strongly support the hypothesis that MELK, unlike other family members, functions by regulating the cell cycle, rather than cell survival by itself, possibly through the regulation of the B-myb proto-oncogene. MELK is also required for the transition of GFAP-positive progenitor cells to highly proliferative GFAP-negative cells in vitro. These data validate our general approach and demonstrate an important role for MELK in neural progenitor biology.

Results

MELK is expressed by neural progenitors

Because MNP characteristics depend upon the age at which they are isolated, producing neurons earlier and glia at later developmental times (Qian et al., 2000; Irvin et al., 2003), we analyzed MELK expression in neurospheres derived from different aged animals. MELK was expressed by NS from embryonic day 12 (E12) telencephalon, as well as E17 and P0 cortex (Fig. 1 A, a). After growth factor withdrawal, MELK mRNA levels declined dramatically, to <10% of the original expression after 24 h (Fig. 1 A, b). MELK was also expressed in NS derived from adult striatal SVZ (unpublished data).

MELK expression declined as progenitor differentiation proceeded, whether the differentiation was induced by growth factor withdrawal or addition of retinoic acid (Fig. 1 B). NS differentiation was confirmed by increased expression of neurofilament heavy chain (NFH), GFAP, and proteolipid protein (PLP)—markers for neuronal, astrocytic, and oligodendroglial differentiation, respectively.

MELK mRNA expression in GZs in vivo

RT-PCR analysis shows that MELK mRNA was expressed in the developing brain during early and mid-embryonic periods with a dramatic decline between E15 and E17, with no detectable expression in adult whole brain or lung (used as a control tissue) (Fig. 2 A). MELK expression in ES cells was relatively high.

In situ hybridization (Fig. 2 B) demonstrated that MELK mRNA was expressed throughout the central nervous system (CNS) within periventricular GZs as early as E9. This general pattern of expression persisted through early postnatal periods to adulthood, including cells of the anterior subventricular zone (SVZa) and rostral migratory stream (Fig. 2 B, b–h). No specific hybridization was detectable in the CNS outside of GZs, indicating that MELK is not expressed by mature cell types. In the adult brain, the only hybridization found was in the SVZ lining the lateral ventricle (Fig. 2 B, h) along its entire rostrocaudal extent, but within only a minority of SVZ cells along the lateral side of the lateral ventricle (Fig. 2 D, arrows). No labeling was detected in adult hippocampus (HC) (Fig. 2 D, a and b) or other GZs. Lack of detection of MELK in hippocampus was further confirmed by RT-PCR (Fig. 2 C).

To further define cell types that express MELK, we performed double labeling with in situ hybridization and immunohistochemistry (Fig. 3). In the brain, MELK was expressed by proliferating cell nuclear antigen (PCNA)–positive cells (Fig. 3 A, a–e). Outside the brain (in the same sections) we did not detect MELK mRNA in PCNA-positive cells, indicating that MELK is not universally expressed by dividing cells (Fig. 3 A, f).

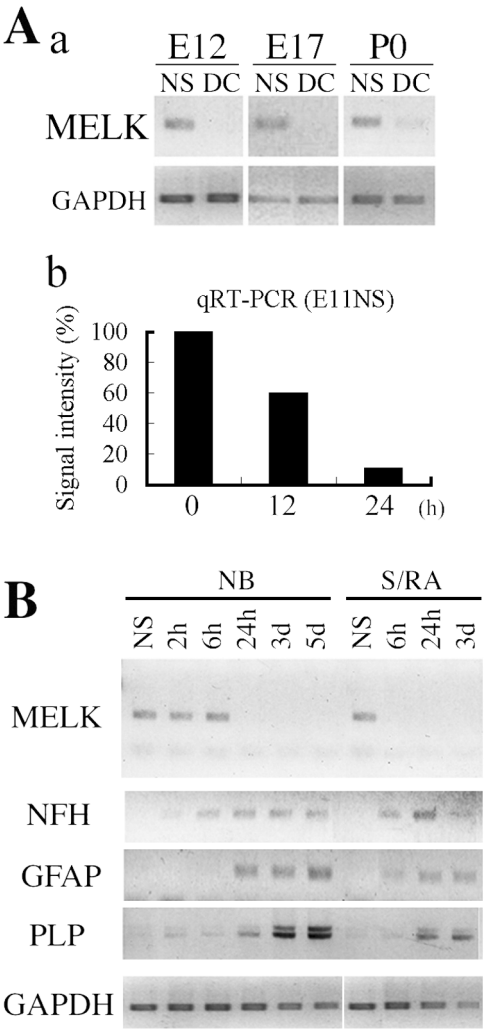

Figure 1. **MELK is highly enriched in cultures containing multipotent progenitors.** (A, a) MELK expression as determined by semiquantitative RT-PCR using GAPDH as a standard in neurospheres (NS) and differentiated sister cultures generated by the withdrawal of bFGF (DC) derived from telencephalon (E12) or cerebral cortex (E17, P0). (b) Quantitative RT-PCR of MELK expression during differentiation of neurospheres derived from E11 telencephalon. (B) RT-PCR analysis of MELK, and lineage-specific markers during E12 neurosphere (NS) differentiation induced by mitogen withdrawal or stimulation of retinoic acid and FBS at the times indicated. Abbreviations: NFH, neurofilament heavy chain; GFAP, glial fibrillary acidic protein; PLP, proteolipid protein.

MELK was also expressed by GFAP-containing cells, although the extent of this colocalization was dependent on the developmental stage. Throughout embryonic and early postnatal ages, MELK-expressing cells were GFAP-negative (Fig. 3 B, a and b, insets) because there is little or no SVZ GFAP expression at these ages (Imura et al., 2003; Fox et al., 2004). Subsequently, as GFAP expression increased in the SVZ, MELK mRNA was detected in some SVZ GFAP-expressing cells. In the adult SVZ, MELK expression was also detectable in GFAP-positive cells (Fig. 3 B, inset in c). MELK, unlike the adult case, was expressed in the hippocampus during early postnatal ages, at least up to P7, within GFAP-positive cells at the hilar border of the dentate gy-

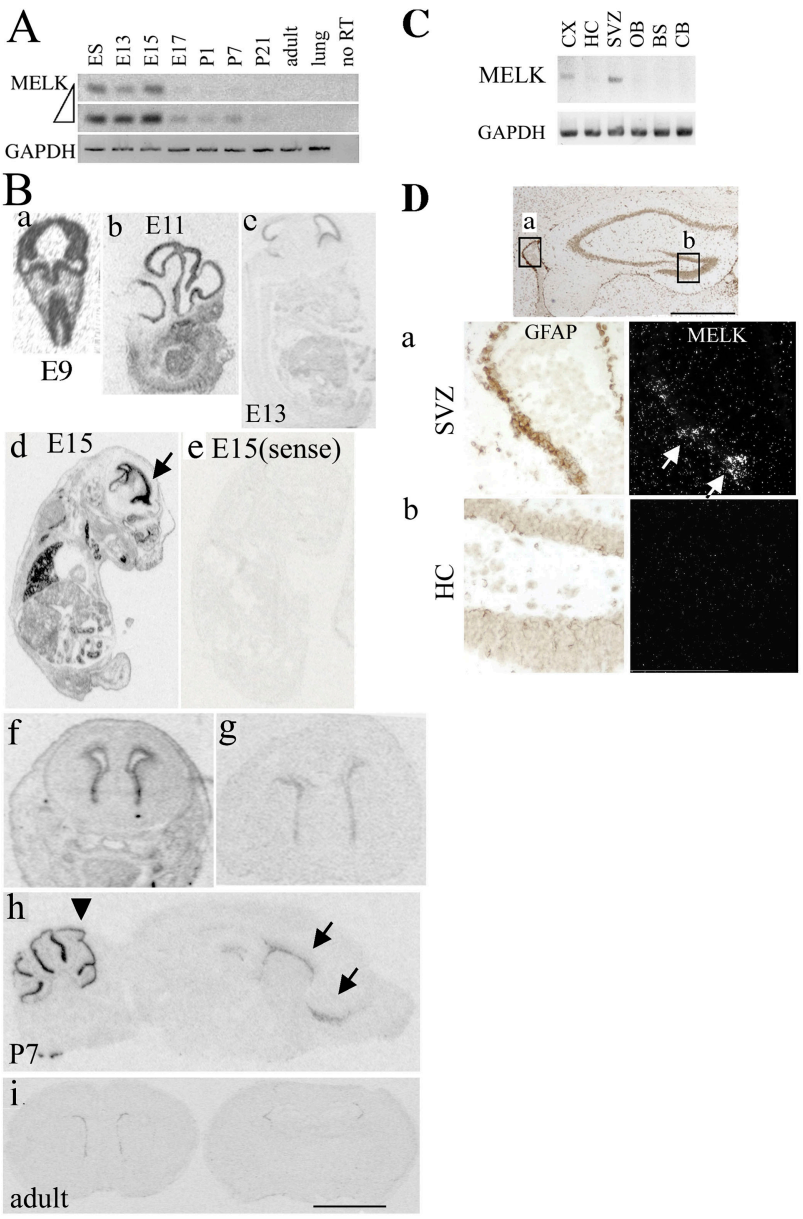

Figure 2. **Developmental and regional expression of MELK mRNA in vivo.** (A, a) MELK expression in ES cells and during brain development analyzed by RT-PCR. The triangle indicates increasing cycle number. (B) In situ hybridization with radiolabeled antisense MELK cRNA. Arrows in indicate the neuroepithelium. Arrowhead in h points to the cerebellum. (e) Sense probe. (C) RT-PCR analysis of different regions of adult brain. (D) Emulsion-dipped brain section (counterstained by GFAP immunohistochemistry) demonstrating hybridization in scattered cells within the forebrain SVZ (a, arrows), but absence of MELK hybridization in the hippocampus (b). Abbreviations: CX, cerebral cortex; HC, hippocampus; OB, olfactory bulb; BS, brain stem; CB, cerebellum. Bar in B: 13.7 mm in a; 8.9 mm in b; 5.5 mm in d–f; 4.1 mm in g; 7.8 mm in h; and 4.5 mm in i. Bar in D: 750 μm in top; 75 μm in a; 75 μm in b.

rus (Fig. 3 C, inset in a). TuJ1-positive neurons in the dentate gyrus or anywhere else did not express MELK (Fig. 3 C, inset in b).

MELK mRNA was also identified within the external granule cell layer (EGL) of the developing cerebellum (Fig. 3 D) within the outer proliferative EGL with no expression in the inner, premigratory, TuJ1-positive zone (Fig. 3 D, c). Expression in the EGL was detectable as early as the EGL could be distinguished clearly at E13 (unpublished data), and disappeared along with the EGL during later postnatal development.

The MELK promoter lies upstream of its first exon, and is active only in undifferentiated neural progenitors

The isolation and initial characterization of a 3.5-kb mouse and human MELK promoter (PMELK) is described in the online supplemental data (available at http://www.jcb.org/cgi/content/full/jcb.200412115/DC1). To investigate the specificity of the PMELK sequence, cells were transfected with PMELK-EGFP or control vectors and then sorted based on EGFP expression. RT-PCR analysis was used to detect MELK expression both in EGFP-positive and -negative populations (Fig. 4 A). The PMELK-EGFP–positive fraction was highly enriched for MELK mRNA as compared with the EGFP-negative fraction or unsorted cells (Fig. 4 A, c).

Using the PMELK-EGFP construct, we characterized the cellular specificity of MELK expression in cortical progenitors derived from E12 embryos (Fig. 4 B). Cells expressing EGFP driven by the CMV promoter were morphologically heterogeneous, whereas MELK promoter-driven EGFP-positive cells were relatively homogeneous with a fusiform shape (Fig. 4 B). MELK-positive cells expressed the neural progenitor markers nestin, NG2, RC2, BLBP, and SOX2 in proliferating cultures (Fig. 4 B, a–o), but no PMELK-driven EGFP was detected in cells expressing differentiation markers (TuJ1, neurons,

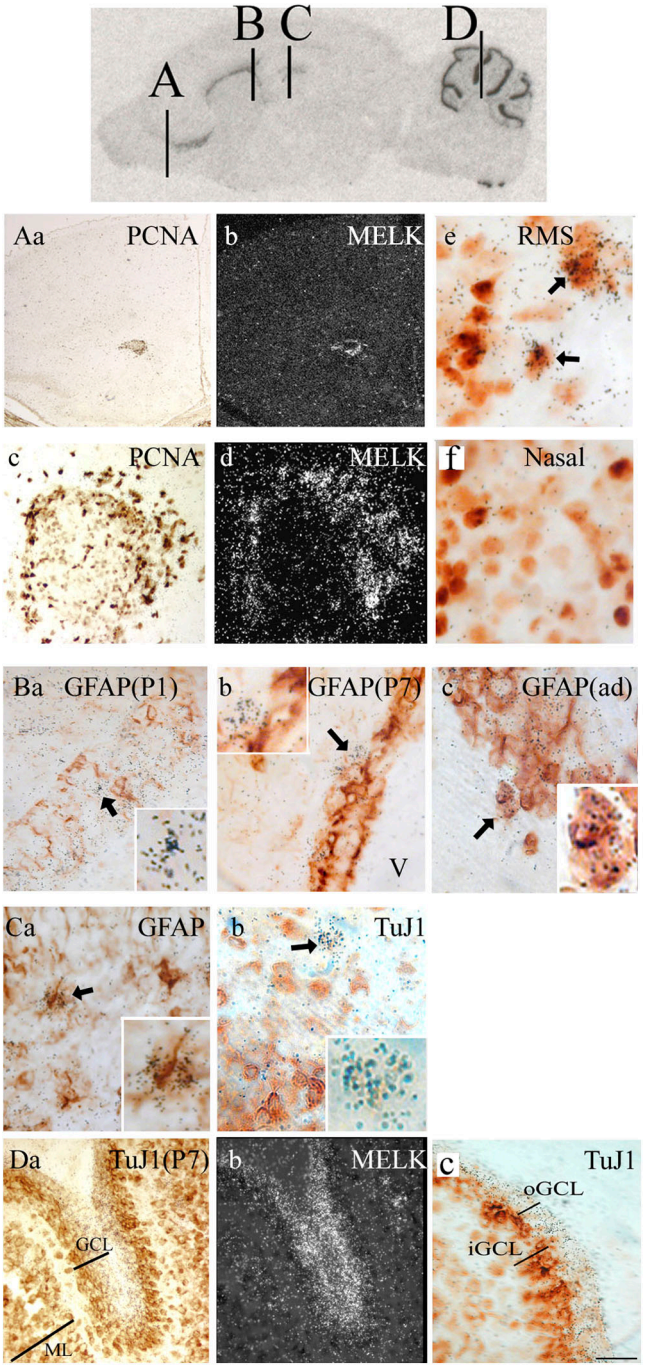

Figure 3. **MELK expression in proliferating CNS progenitors in vivo.** The photomicrographs in A–D are of dipped sections sampled from the regions identified in the brain section at the top. Sections were hybridized with MELK cRNA, and then stained by immunohistochemistry. (A) Coronal section through the rostral forebrain at P7. MELK mRNA was restricted to the germinal epithelium (a and b). Co-expression in brain GZs with PCNA (a–d, arrows in e). MELK was not detected in extracranial PCNA-positive cells (f). (B) Limited to no coexpression with GFAP on P1 or P7 (a and b, arrows), with greater coexpression in adult SVZ (c, arrow). (C) Co-expression with GFAP in the hippocampus hilar border on P7 (a, arrow). There is no coexpression with TuJ1 in the dentate gyrus (b, arrow). (D) Granule cell layer expression in the P7 cerebellum (a and b). MELK mRNA was present in the outer proliferative region, but not in the inner premigratory TuJ1-positive granule cells (c). Cells indicated by arrows are shown in the insets. GCL, granule cell layer; iGCL, inner layer of GCL; oGCL, outer layer of GCL; V, ventricle. Bar in A: 610 μm in a and b; 61 μm in c and d; 22 μm in e and f. bar in B: 55 μm in a; 45 μm in b; 31 μm in c. Bar in C: 22 μm. Bar in D: 50 μm in a and b; 22 μm in c.

GFAP, astrocytes and O4, oligodendrocytes), even in proliferating cultures (Fig. 4 B, p–v). These data indicate that the MELK promoter is active only in neural progenitors, and not in more differentiated cells. Furthermore, the data are consistent with, and support the findings of, native MELK expression described above.

MELK is a marker for tripotent, self-renewing progenitors in embryonic cortical cultures

MNPs have the fundamental properties of self-renewal and multipotency. Therefore, we tested the ability of MELK-expressing cells to form primary and secondary neurospheres and examined the differentiation capacity of these spheres. The LeX antigen is expressed by neural progenitors, and LeX-positive cells form neurospheres (Capela and Temple, 2002). Immunocytochemistry shows that virtually all EGFP-expressing cells also expressed LeX (Fig. 5 A). Cultures from E12 telencephalon were then separated by FACS using an anti-LeX antibody (Fig. 5 B). Approximately 65% of the cells in the cultures were LeX-positive (Fig. 5 B, a and b). RT-PCR analysis demonstrated that MELK mRNA was completely restricted to the LeX-positive fraction (Fig. 5 B, c). LeX sorting also resulted in enrichment of other neural stem cell–associated genes, including nucleostemin (NCS) and SOX2. In contrast, musashi1 (Msi1) and GFAP were not enriched in the LeX-positive fraction (Fig. 5 B, c), consistent with previous observations of their expression in both progenitor and nonprogenitor populations (Kaneko et al., 2000).

We next tested the capacity of MELK-expressing cells to form neurospheres. MELK-positive E15 progenitors generated ~5 times more primary neurospheres than LeX-positive cells at a density (2,000 cells/ml) where most spheres form from a single cell (Tropepe et al., 1999) (Fig. 5 C). Given that virtually all MELK-positive cells express LeX, these data suggest that the MELK-positive fraction of LeX-expressing cells is more highly enriched for sphere-initiating cells. LeX-negative populations did not generate neurospheres under these conditions. Primary spheres derived from MELK-positive progenitors formed "secondary" neurospheres when dissociated and replated (Fig. 5 C, g), indicating self-renewal capacity. Control cultures transfected with cytomegalovirus promoter (PCMV)-EGFP yielded equivalent percentages of neurospheres in EGFP-positive and -negative fractions (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200412115/DC1), indicating that the present findings are not simply due to a general preference for transfection of sphere-forming cells.

To more accurately determine the frequency of neurosphere-initiating cells (NS-ICs), sorted progenitors from E15 telencephalon cultures were serially diluted. At each density, MELK-positive progenitors gave rise to significantly greater numbers of spheres than did LeX-positive progenitors. Approximately 1 out of 10 MELK-positive progenitors were NS-IC, whereas 1 out of 29 LeX-positive cells was NS-IC (Fig. 5 C, h and i). Thus, even at an extremely low seeding density, MELK-expressing cells were highly enriched for NS-IC.

Staining of undifferentiated MELKP-EGFP–derived neurospheres revealed that virtually all cells expressed nestin and

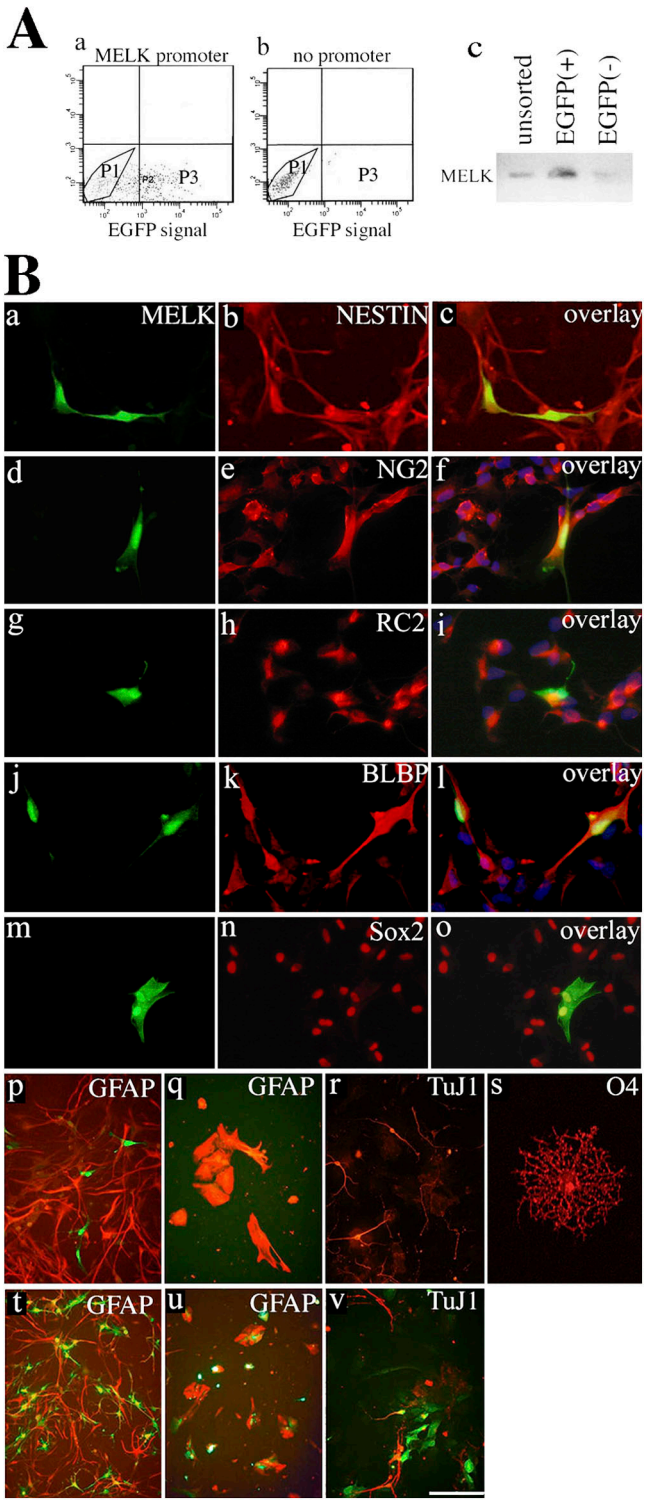

Figure 4. **The MELK promoter is active only in undifferentiated neural progenitors.** (A) FACS analysis of UD cells transfected with the MELK promoter–containing (a) and control (b) EGFP clones. (c) RT-PCR for MELK after separation of the fluorescence-positive (P3) and -negative (P1) cells in panel a by flow cytometry. (B) Colocalization of EGFP fluorescence driven by the MELK promoter (a–p) or the CMV promoter (t) in undifferentiated progenitors or in differentiated progenitors (q–s, u, and v) with nestin, NG2, RC2, BLBP, Sox2, GFAP, O4, or TuJ1 immunoreactivity. Bar in B: 44 μm in all except for s, where it equals 22 μm.

LeX (Fig. 5 D, a and b). After differentiation of primary or secondary spheres, staining revealed that the spheres formed neurons, astrocytes, and oligodendrocytes (Fig. 5 D, c–e). These data demonstrate that MELKP-derived cells are indeed multipotent, self-renewing progenitors.

MELK is expressed by self-renewing multipotent progenitors in vivo

To examine whether MELK can be expressed by MNP in vivo, we constructed transgenic reporter mice using the MELK promoter to drive EGFP expression. In general, EGFP expression recapitulated the expression pattern of endogenous MELK mRNA being largely restricted to developing GZ, including the GZ surrounding the lateral ventricles and the rostral migratory stream, the inner granule zone of the early postnatal hippocampus, and external granule cells of the neonatal cerebellum (Fig. 6 A, b–e).

Cortical progenitors from P1 transgenic mice were cultured as neurospheres according to the schemes shown in Fig. 6 A (a). Primary neurospheres were all EGFP positive (Fig. 6 A, f). After up to 12 clonal passages over 4 mo, neurospheres remained EGFP positive (Fig. 6 A, g and h). EGFP-positive neurospheres derived from the MELK-EGFP transgenic mice were multipotent, containing neurons, astrocytes, and oligodendrocytes after induction of differentiation at each passage (Fig. 6 A, f). These findings indicate that MELK expression persists in progenitors within clonally passaged neurospheres throughout multiple rounds of self-renewal. To determine whether sphere-initiating progenitors are EGFP positive, we performed FACS for EGFP and then grew neurospheres at high and clonal densities from P1 forebrain. As is shown in Fig. 6 B (b), EGFP-expressing cells yielded neurospheres both in clonal and high density conditions. In contrast, MELK-negative progenitors failed to form neurospheres even in high density conditions. Thus, neurosphere-forming cells derived from the developing brain express MELK, and MELK expression persists throughout multiple passages, suggesting that it is expressed by long-term, self-renewing progenitors.

MELK regulates MNP proliferation

The studies thus far demonstrate that MELK is expressed by MNPs. To determine the function of MELK in these cells, we assessed the effects of overexpression and knockdown according to the scheme shown in Fig. 7 A. Neurospheres were generated from the following: E12 telencephalon as a stage of neurogenesis, E15 and P0 cerebral cortex as stages of transition and gliogenesis, respectively. Adherent cultures of progenitors derived from neurospheres were transduced with expression vectors or appropriate double-strand RNA designed to be small inhibitory RNA (siRNA) or controls. Using PCMV-EGFP we estimated transfection efficiency at ~70% (unpublished data). Specificity and efficacy of the overexpression and siRNA vectors used is described in the online supplemental data, and illustrated in Fig. S3 (available at http://www.jcb.org/cgi/content/full/jcb.200412115/DC1). In addition to mock transfection, we used NCS and CRT1 siRNAs as positive and negative controls, due to previous studies demonstrating that NCS promotes MNP proliferation, whereas CRT1 does not (Rauch et al., 2000; Tsai and McKay, 2002). These adherent cultures varied in their char-

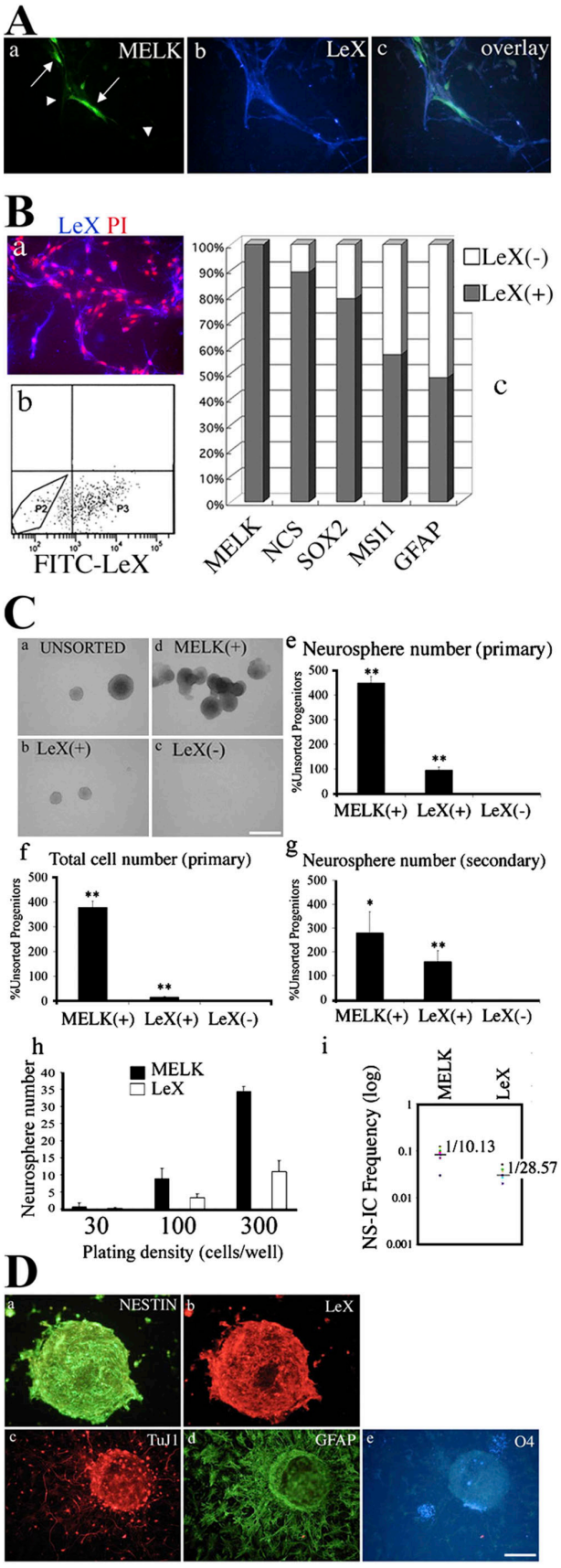

Figure 5. **MELK-expressing progenitors are neurosphere-initiating MNPs.** (A) PMELK-EGFP expression overlaps with LeX immunofluorescence. Arrows/arrowheads indicate LeX-positive, MELK-negative cells in the same culture. Arrowhead is negative, arrow is positive. (B) MELK expression in LeX-sorted cells. (a) Staining of sorted cells (P3 fraction in b) with anti-LeX antibody. (b) FACS analysis showing LeX-positive (P3) and -negative (P2) fractions. (c) Percentage of total RT-PCR product in Lex-positive (gray bar) vs. LeX-negative (white bar) fraction for each gene listed. 100% is the total amount of GAPDH-normalized signal in LeX-positive and LeX-negative fractions combined. (C) Neurospheres production after LeX sorting or sorting for GFP after transfection of PMELK-EGFP (a–d). (e) Neurosphere numbers obtained from sorted cells expressed as a percentage of cells obtained in unsorted populations. (f) Cell numbers corresponding to the conditions in panel e. (g) Secondary neurosphere numbers after dissociation of the primary spheres counted in panel e as a percentage of the primary neurosphere numbers derived from unsorted progenitors. The graph in panel h demonstrates the numbers of neurospheres resulting from the seeding of 30, 100, or 300 cells, achieved by serial dilution, of MELK-positive cells and LeX-positive cells. (i) Frequency of neurosphere-initiating cells (NS-IC). (D) Secondary neurospheres from MELK-positive progenitors were stained as spheres (top panels) or after differentiation in the absence of mitogen. Undifferentiated spheres intensely labeled with anti-nestin and anti-LeX antibodies (a and b). Differentiated spheres demonstrate TuJ1-positive neurons, GFAP-positive astrocytes, and O4-positive oligodendrocytes (c–e). Asterisk denotes different from controls, $P < 0.05$; **, $P < 0.001$, ANOVA followed by post-hoc *t* test. Bar in C: 200 μm. Bar in D: 110 μm in a and b; 207 μm in c–e.

acteristics, depending on age. E12 telencephalic cells largely contained nestin/LeX-positive cells, with few cells bearing differentiation markers, whereas cultures from older animals contained more cells expressing differentiation markers (Fig. 7 B, a–g). Spheres were generated from transfected cultures and propagated. To assay sphere potency, we differentiated E12-derived spheres by removal of growth factor and plating on substrate, and found that they reliably and readily formed neurons, astrocytes, and oligodendrocytes (Fig. 7 B, h–j).

Overexpression of MELK in neural progenitors yielded increased numbers of spheres after transfection. MELK knockdown resulted in the opposite effect: a diminished number of spheres compared with controls, indicating that MELK regulates the proliferation of sphere-forming progenitors (Fig. 7 C, a–c). As expected, knockdown of NCS had effects similar to that of MELK siRNA, whereas knockdown of CRT1 had no effect. The total number of cells within cultures was affected as well, with MELK overexpression resulting in a greater number of cells and knockdown in fewer cells. MELK overexpression resulted in significantly larger spheres, compared with control conditions or siRNA for MELK (Fig. 7 C, e). This latter finding suggests that MELK overexpression influences not only sphere-initiating cells, but also cells that contribute to overall sphere size.

MELK knockdown inhibited (whereas overexpression enhanced) BrdU labeling indices after pulse labeling, indicating a direct effect on proliferation (Fig. 7 C, f). The number of dead or dying cells was not affected by siRNA treatment (Fig. 7 C, g). These data suggest that MELK influenced proliferation itself rather than survival of proliferating cells.

Spheres generated after MELK knockdown or overexpression were multipotent, yielding neurons, astrocytes (Fig. 7 D), and oligodendrocytes (not depicted). The neurogenic capacity was not significantly altered by the change of MELK expression, indicating that endogenous MELK likely regulated the proliferation of sphere-forming cells, which were, in turn, multipotent, without influencing the relative numbers of differentiated cells (i.e., the proliferation of committed progenitors). To determine whether MELK directly

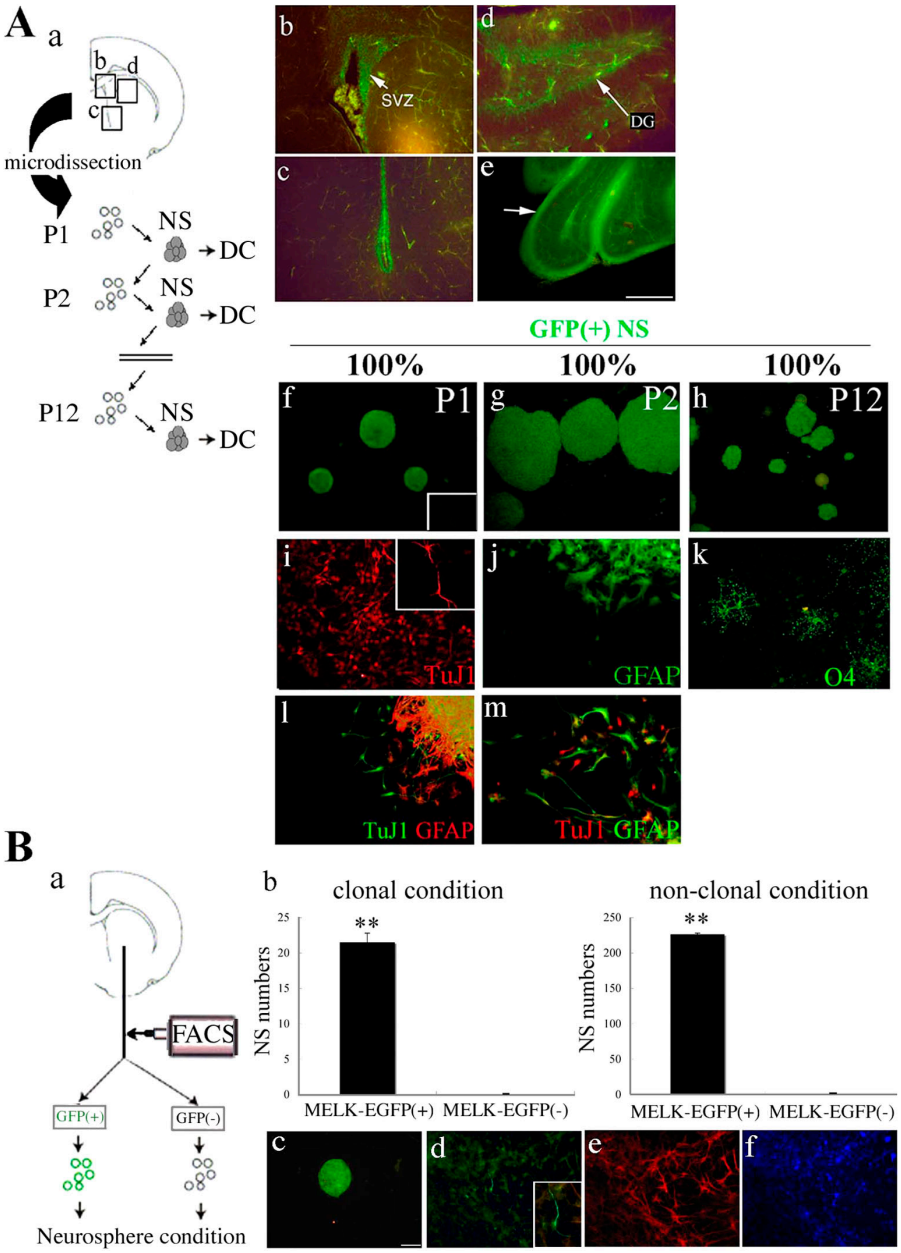

Figure 6. **MELK-positive cells are self-renewing multipotent progenitors in vivo.** (A, a) Experimental design of long-term passage of neurospheres from transgenic reporter mice. Expression of EGFP in a P8 transgenic mouse demonstrating specific signals in the SVZ (b and c), dentate gyrus (DG) of hippocampus (d), and granule cell layer in cerebellum (e), all indicated by arrows. (f–m) Neurospheres grown from P1 cortices were substantially all EGFP-positive from passage 1 (P1, f) to passage 12 (P12, h). Inset in (f) shows a wild-type neurosphere. Differentiated primary spheres contain TuJ1-positive neurons (i), GFAP-positive astrocytes (j), and O4-positive oligodendrocytes (k). Inset in (i) shows a magnified positive cell. Differentiated cells from P2 and P12 neurospheres are shown by immunocytochemistry (l and m). (B, a) Experimental design of direct FACS from P1 cortex. (b) Graph shows the number of neurospheres from PMELK-EGFP(+) and PMELK-EGFP(−) cells both in clonal and nonclonal conditions. **, $P < 0.001$, ANOVA followed by post-hoc *t* test. Undifferentiated clonal neurospheres (c) were differentiated and stained with TuJ1 (d), GFAP (e), and O4 (f). Inset in (d) shows a magnified positive cell. Bar in A: 375 μm in b and c; 188 μm in d and e; 44 μm in f–j and l; 88 μm in k and m. Bar in B:110 μm in c; 44 μm in d–f.

influences differentiation, we analyzed the effects of MELK knockdown and overexpression in adherent E12 cortical progenitors that were then differentiated on the coverslip for 5 d by withdrawal of bFGF, and found no effect on the formation of neurons, astrocytes (Fig. 7 D, c and d), or oligodendrocytes (not depicted). Together, these functional experiments indicate that MELK regulates MNP proliferation and their capacity to self-renew, at least in the short term, without a major effect on the proliferation of committed progenitors or on cell fate decisions.

MELK is necessary for the production of GFAP-negative MNPs from neonatal astrocyte cultures

Recent studies have documented the ability of GFAP-positive cells of the adult forebrain SVZ to form rapidly amplifying progenitors in the presence of bFGF (Imura et al., 2003; Morshead et al., 2003). These transition processes can be monitored by RT-PCR and immunocytochemistry (Fig. 8, A and B). 24 h of bFGF treatment resulted in diminished GFAP mRNA expression and increased NCS expression. MASH1 mRNA was up-regulated after 7 d, but not 24 h of treatment (Fig. 8 A). On d 0, virtually all the cells in culture were GFAP immunoreactive, whereas a minority (~5%) was strongly LeX positive (Fig. 8, B and C). 5 d after placement in bFGF, GFAP immunoreactivity had dramatically declined, and ~30% of the total cell numbers were strongly LeX positive (Fig. 8 B and Fig. 8 C, b). Most of these LeX-positive cells were either GFAP negative or weakly GFAP positive. These LeX-positive cells function as progenitors, as the number of neurospheres produced from the LeX-positive fraction, after 2 d of bFGF treatment, was markedly higher than the number

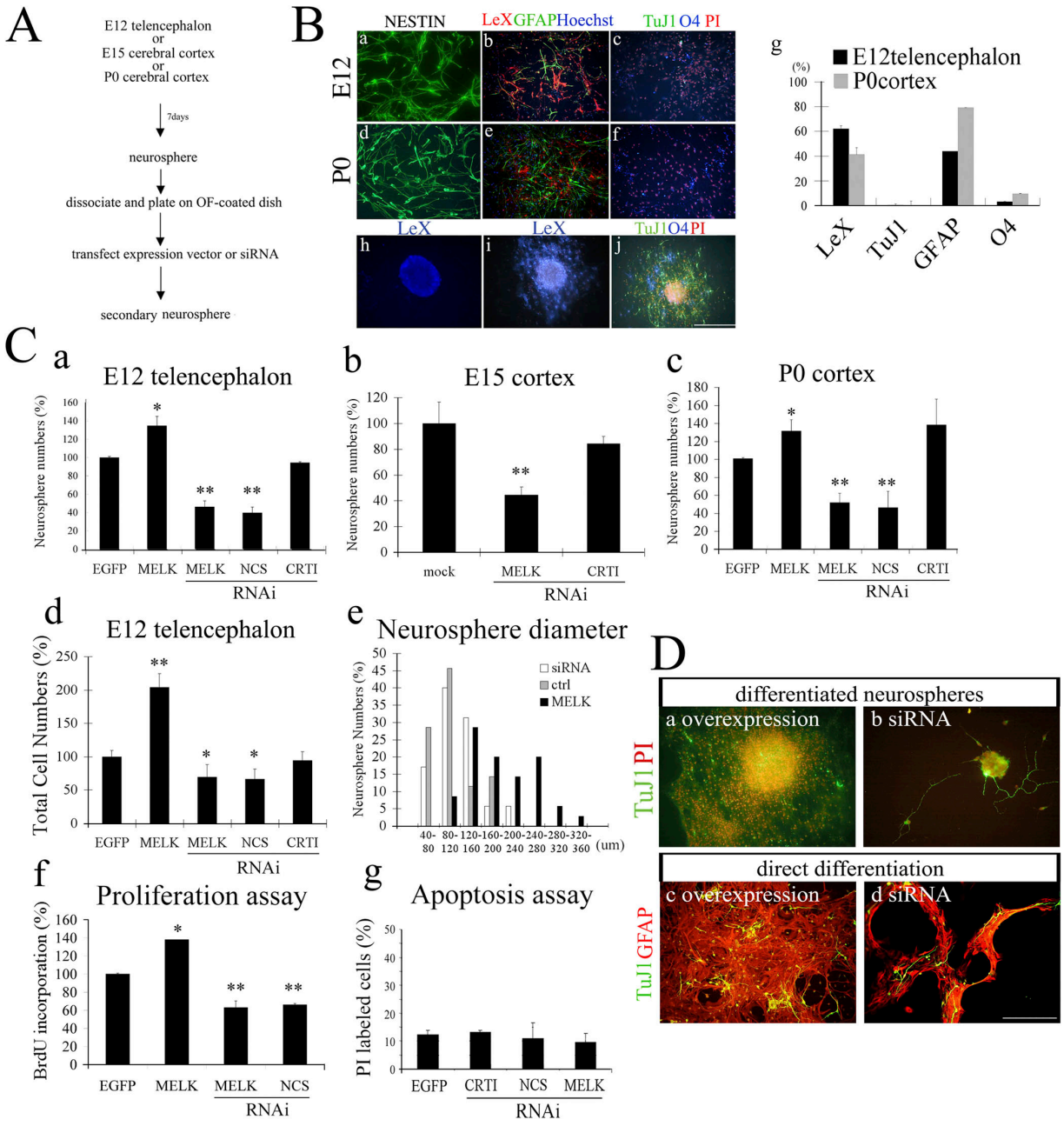

Figure 7. **MELK regulates neural progenitor proliferation.** (A) Experimental design. (B) Characterization of adherent progenitors from neurospheres generated from E12 telencephalon and P0 cerebral cortices (a–f). Monolayer progenitor cultures from neurospheres were immunostained for nestin, LeX, GFAP, TuJ1, and O4 antibodies. Propidium iodide (PI) was used for nuclear staining. (g) Relative percentages of adherent cells expressing markers. (h) LeX staining of undifferentiated secondary spheres. (i and j) LeX, TuJ1, O4, and PI staining of differentiating secondary spheres (C) Sphere counts (a–c), total cell counts (d), sphere diameters (e), percent BrdU incorporation (f), and percent apoptotic cells (g) after overexpression or knockdown of MELK in adherent progenitors cultured at the ages shown. Controls cultures were transfected with EGFP-expressing cDNA or nucleostemin (NCS) or calreticulin (CRT1) siRNAs. All graphs are the means ± SD. (D) TuJ1 immunoreactivity of differentiating secondary neurospheres, which were derived from primary neurospheres after transfection (a and b) and of adherent progenitors from primary E12 neurospheres that were directly differentiated (c and d) after transfection. Asterisk denotes different from controls, $P < 0.05$; **, $P < 0.001$, ANOVA followed by post-hoc *t* test. Bar in B: 110 μm in a–f; 215 μm in h–j. Bar in D: 235 μm in all panels.

from the LeX-negative fraction (Fig. 8 C, a), and the LeX-positive cell-derived spheres were competent to produce neurons in addition to glia (unpublished data). Together, these findings are consistent with the hypothesis that the addition of bFGF to these cultures results in the production of highly proliferative, GFAP-negative, LeX-positive MNPs from GFAP-positive cells.

After bFGF treatment MELK mRNA expression was up-regulated, whereas GFAP expression declined (Fig. 8 A). These observations suggest that high levels of MELK expression is either a reflection of the MNP state or that MELK regulates the production of, or transition to, GFAP-negative/LeX-positive cells. To determine whether this transition was dependent on MELK, we decreased MELK expression during

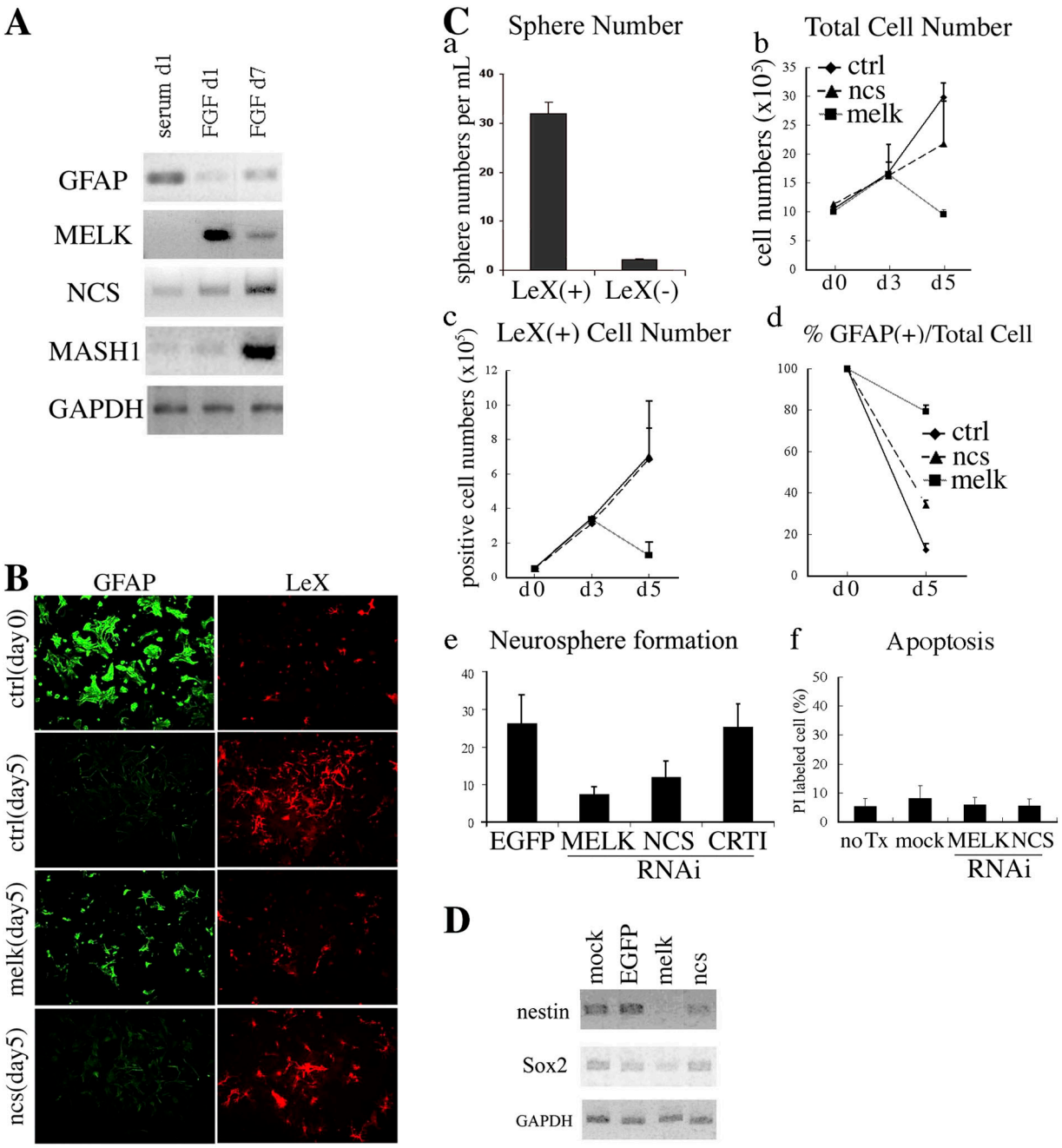

Figure 8. **MELK is necessary for the transition from GFAP-positive into GFAP-negative highly proliferative progenitors in vitro.** (A) RT-PCR of cortical astrocyte cultures after addition of bFGF on d 1 and 7. (B and C) Effects of MELK siRNA on the formation of multipotent progenitors from astrocyte cultures. (B) Immunostaining for GFAP and LeX after bFGF addition. (C, a) Sphere-forming frequency of LeX-positive and -negative cells derived from GFAP-positive astrocyte cultures (mean ± SD). Change in total cell number (b), LeX-positive cell number (c), GFAP-positive cell number (d), neurosphere numbers (e), and apoptosis (f) after MELK siRNA or control treatment in bFGF-treated astrocyte cultures. Counts were based on two independent experiments for each condition (ctrl, control; crt1, calreticulin1 siRNA; NCS = nucleostemin siRNA). (D) RT-PCR analysis of cultures, after MELK siRNA or control treatment.

bFGF stimulation. Strikingly, siRNA for MELK, but not for NCS, resulted in diminished numbers of neurospheres (Fig. 8 C, e) and prevented the increase in numbers of LeX-positive cells (Fig. 8 B and Fig. 8 C, c). Instead, there was a relative persistence of GFAP-positive cells (Fig. 8 C, d). Knockdown of MELK also resulted in the reduced expression of nestin and SOX2 during bFGF treatment (Fig. 8 D). However, knockdown did not influence cell survival (Fig. 8 C, f). These data show that MELK is necessary for the production of GFAP low or negative, LeX-positive MNPs from progenitors that highly express GFAP.

MELK expression is cell cycle-regulated and MELK function is likely mediated through the B-myb proto-oncogene

Our data thus far indicate that MELK plays an important role in neural progenitor proliferation. This was somewhat surprising because other members of the snf1/AMPK family appear to

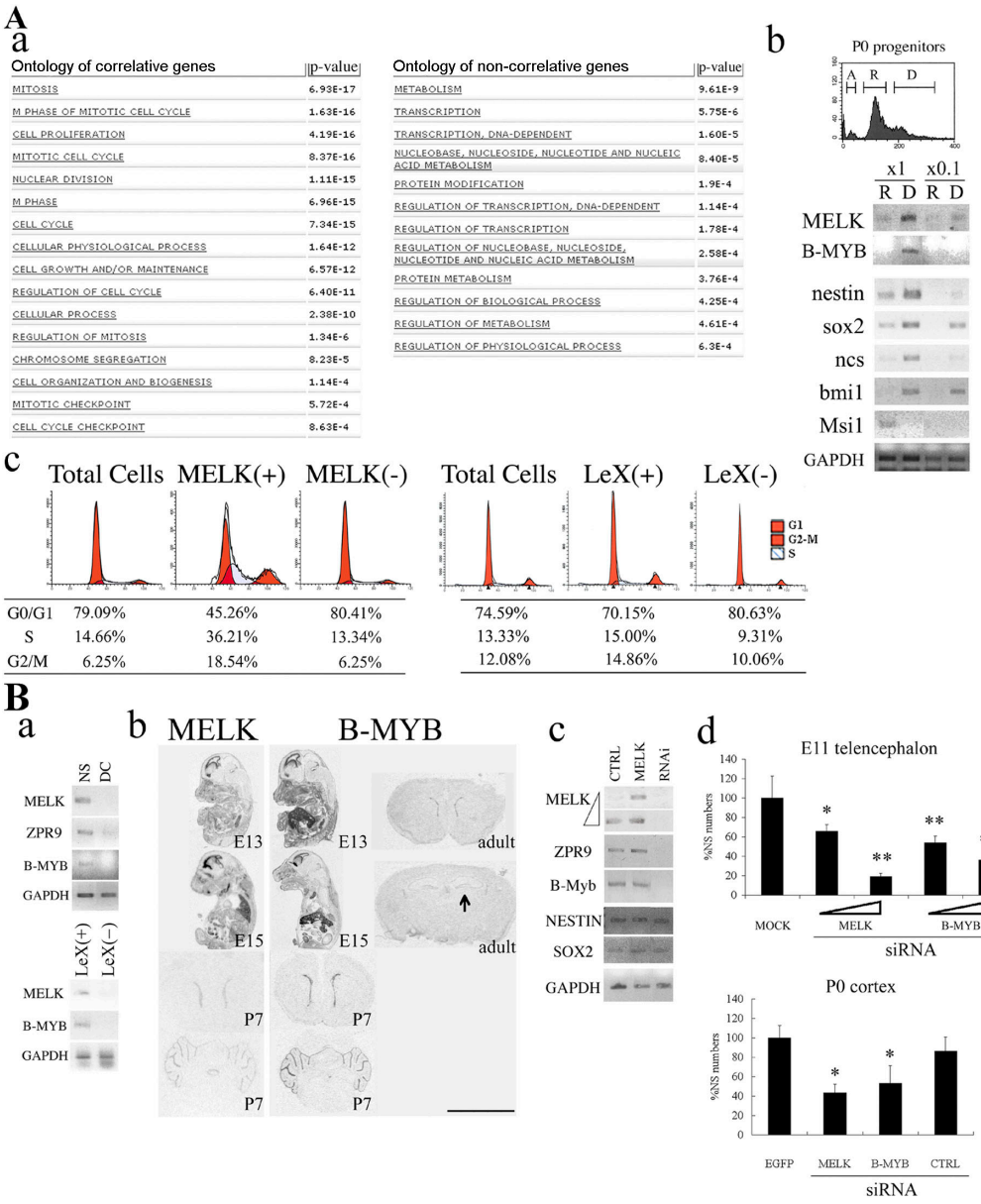

Figure 9. **Cell cycle regulation and the B-myb proto-oncogene in MELK function.** (A, a) Functional grouping of genes most and least correlated with MELK expression; $P < 0.001$. (b) RT-PCR after separation of P0 progenitors into apoptotic (A), G0/G1 phases (R), and S/G2/M phases (D). Top panel shows FACS of P0 neurospheres using PI-stained cells. (c) Cell cycle analysis after separation based on either MELK (using PMELK-EGFP) or LeX expression. (B, a) Top: RT-PCR of P0 neurospheres (NS) and differentiated neurospheres (DC). Bottom: RT-PCR after LeX sorting of progenitors derived from E12 telencephalon. (b) B-myb and MELK in situ hybridization. Arrow in adult section indicates expression in the adult hippocampus. (c) RT-PCR after either overexpression or siRNA knockdown of MELK using telencephalic progenitors derived from E11 animals. Triangle indicates increase in PCR cycle number. (d) Effect of B-myb knockdown on neurosphere generation. For E11 progenitors, siRNA concentrations were 25 and 100 nM (triangles). For the P0 progenitors, 100 nM was used. Graph shows the ratio of neurosphere formation for each condition compared with the control condition (mock-transfected on the left, calreticulin siRNA on the right). Asterisk = different from controls, $P < 0.05$; **, $P < 0.001$, ANOVA followed by post-hoc *t* test. Abbreviations: ncs, nucleostemin; msi1, musashi1. Bar in B: 4.5 mm.

function in cell survival (Kato et al., 2002; Inoki et al., 2003; Suzuki et al., 2003a,b). To further explore potential roles that MELK may play in cellular function, we used a large microarray dataset derived from human brain tumors to identify genes whose expressions were coregulated with MELK. MELK expression was highly and significantly correlated with genes known to play roles in the cell cycle, especially those associated with the mitosis (M) phase. (Fig. 9 A, a). Genes whose expression was not correlated with MELK functioned in other processes as determined by Gene Ontology, including metabolism, transcription, and protein modification. Thus, this genome-wide analysis of coregulation supports a role for MELK in cell cycle regulation.

Many genes that play roles in the cell cycle show phase-dependent transcriptional regulation. Therefore, we sorted progenitors based on their DNA content and evaluated the expression of MELK and other progenitor genes. MELK, like nestin, Sox2, and bmi-1, but unlike Msi1, was most highly

expressed during phases of the cell cycle with 4n DNA content (S, G2, and M) rather than at G0–G1, indicating that MELK expression varies with the cell cycle (Fig. 9 A, b). To examine the cell cycle characteristics of MELK-expressing cells, we transfected progenitor cultures with the PMELK-EGFP or control construct and analyzed cell cycle parameters by FACS (Fig. 9 A, c). Greater numbers of MELK-expressing cells were found to be in the S and G2/M phases, whereas fewer were in the G0/G1 phase compared with the total or putative non-MELK–expressing cells. On the other hand, LeX-positive cells were not different from the total cell fraction or LeX-negative cells in their cell cycle parameters.

The data described in this section and those above indicate that MELK functions in the regulation of the cell cycle in MNPs. However, its mechanism is unknown. Previous studies have demonstrated the importance of the PTEN/AKT/MTOR pathway in MNP proliferation (Groszer et al., 2001; Sinor and Lillien, 2004). However, as described in Fig. S4 (available at http://www.jcb.org/cgi/content/full/jcb.200412115/DC1), we did not find evidence that MELK interacts with this pathway. Recent studies have implicated the protein ZPR9 in the function of MELK and, in turn, ZPR9 in the function of the cell cycle regulatory proto-oncogene B-myb (Seong et al., 2002, 2003). To determine whether MELK function could be mediated by B-myb in MNP, we first examined Zpr9 and B-myb expression in cultured neural progenitors (Fig. 9 B, a). As is the case for MELK, ZPR9 and B-myb were highly enriched in NS from P0 cortex compared with DC (Fig. 9 B, a, top). Also, like MELK, B-myb was enriched in the LeX-positive fraction of neurospheres (Fig. 9 B, a, bottom). B-myb, like MELK, was also expressed during phases of the cell cycle with 4n DNA content (Fig. 9 A, b). The brain expression pattern of B-myb was similar to that of MELK throughout development, with the exception of the adult hippocampus, where B-myb mRNA was found to be expressed (Fig. 9 B, b).

MELK siRNA treatment resulted in a down-regulation of both B-myb and ZPR9, without significantly influencing other stem cell–related genes such as nestin or SOX2 (Fig. 9 B, c) 48 h after transfection. Knockdown of B-myb produced similar effects to MELK, resulting in a dose-dependent decrease in neurosphere formation from progenitors (Fig. 9 C, d). Thus, these data suggest that inhibition of endogenous MELK expression down-regulates B-myb, which, in turn, results in the reduction of neurosphere numbers and is consistent with the hypothesis that MELK exerts some or all of its actions via regulation of B-myb expression.

Discussion

MELK is a member of the SNF1/AMPK family of serine threonine kinases and its function was previously unknown. Here, we demonstrate that MELK is expressed by and is a marker for self-renewing, tripotent progenitors, MNP, based on in vivo and in vitro studies. Functional studies demonstrate that MELK is critical for MNP proliferation, and that MELK is required for the transition from GFAP-positive progenitors to rapidly proliferative multipotent GFAP-negative progenitors.

MELK in vivo

Our in vitro studies take advantage of the quantitative aspects of neurosphere cultures, a system that allows for the reproducible determination of the numbers of MNP after experimental manipulations. These culture systems, despite their potential shortfalls, can provide significant insight into the function of genes in vivo (Groszer et al., 2001; Molofsky et al., 2003). However, in vitro studies cannot, in isolation, be used as sole evidence of in vivo function, as the in vivo environment places specific constraints on progenitor cells. Therefore, we sought to determine the relevance of our in vitro findings by detailed examination of MELK expression in vivo. MELK expression is limited to areas containing proliferating neural progenitors, the periventricular GZ, the developing hippocampus, and the EGL of the cerebellum. Furthermore, double-labeling studies demonstrate that MELK is expressed by proliferative cells in these areas. Within the embryonic telencephalon, MELK is clearly expressed by self-renewing MNP, as MELK is found throughout the proliferative zones as early as E9, a stage when most of (if not all) the cells are likely to be MNP rather than committed progenitors (Cai et al., 2002). It remains to be seen, however, if the MELK expressed within later GZ is restricted to MNPs or is also expressed by committed progenitors. The expression of MELK in granule cell progenitors suggests that MELK can be expressed by populations of self-renewing, committed progenitors, rather than only MNP.

In the hippocampus, MELK expression was not detectable in the adult dentate gyrus, a site of neurogenesis and presumed location of stem cells (for review see Gage et al., 1998). Thus, MELK expression is neither present in all neural stem cells nor required for the multipotent state. The lack of expression in adult hippocampus does suggest, however, that there are differences between progenitor cells within the hippocampus and the SVZ. One potential explanation is that MELK is expressed in a class of highly proliferative progenitors that are not found in the adult hippocampus. Previous studies have indicated that different types of transitory progenitors are derived from GFAP-positive stem cells in the adult hippocampus and the SVZ (Seri et al., 2001). Additionally, studies have shown differences in neurosphere-forming capacity between cells derived from the dentate gyrus and those derived from the lateral ventricles, again suggesting fundamental differences between progenitors derived from the two regions (Seaberg and van der Kooy, 2002).

MELK is a marker for self-renewing multipotent neural progenitors in the developing forebrain

Here, we demonstrate that MELK expression can be used to prospectively isolate MNP from developing brain. The MELK promoter element drives EGFP expression faithfully, allowing for isolation of MELK-expressing cells by FACS. This approach has been taken using other genes, including nestin, Msi1, and SOX2 (Roy et al., 2000; Keyoung et al., 2001). Using the nestin promoter/enhancer or the Msi1 promoter, others have found that ~1–2% of the isolated, EGFP-

expressing cells form neurospheres (Keyoung et al., 2001). Other non-gene based methods have also been used to enrich for neural stem cells from brain or neurospheres, including size (Murayama et al., 2002) and exclusion of Hoechst dye (Kim and Morshead, 2003; side population), the latter of which yields an approximate 1 in 10 neurosphere formation. Positive sorting using anti-LeX antibody also enriches for neural stem cells in adult brain (Capela and Temple, 2002). In the present study, the relative enrichment for neurosphere initiation with PMELK-EGFP was greater than that for LeX, as well as for previously reported results using other promoters (Roy et al., 2000; Keyoung et al., 2001). There was approximately the same level of enrichment reported using side population purification. The cell-sorting and immunocytochemical data presented here are consistent with the hypothesis that MELK-expressing cells are the subset of LeX-positive cells that form neurospheres.

Our studies with MELK-EGFP transgenic mice indicate that MELK-expressing cells in the developing forebrain in vivo can serve as self-renewing multipotent progenitors. MELK-expressing cells could be cultured as neurospheres that could be multiply passaged to form new neurospheres at clonal densities. These neurospheres could then be differentiated with the resulting production of neurons, astrocytes, and oligodendrocytes. Thus, MELK-expressing cells isolated from the brain contain a population with the characteristics of neural stem cells—persistent self-renewal and multipotency.

MELK is necessary for MNP proliferation in vitro

Here, we demonstrate that MELK is necessary for MNP proliferation from embryonic and early postnatal cortex, a novel function for this putative kinase. Previous studies of other family members in transformed cells have revealed that they largely mediate cell survival under hostile conditions (Kato et al., 2002; Inoki et al., 2003; Suzuki et al., 2003a,b). MELK appears to be unique amongst this family in its capacity to regulate the cell cycle.

In vitro, we see diminished numbers of secondary multipotent neurospheres in MELK siRNA-treated cultures, indicating that MELK is necessary for the self-renewal of MNP, at least in the short term. Part of the definition of stem cells lies in their capacity to self-renew. It is self-renewing divisions that allow for the maintenance of a stem cell pool and is critical to the formation and maintenance of the CNS. During early development, the neural tube consists primarily of MNP undergoing extensive, symmetrical self-renewal. Factors, such as PTEN (Groszer et al., 2001), Bmi-1 (Molofsky et al., 2003), or the Wnt pathways (Chenn and Walsh, 2002) that regulate this process influence ultimate brain size. The methods used here do not allow us to determine whether MELK is required for the long-term self-renewal of neural stem or progenitor cells as opposed to just being active in short-term amplifying progenitors. However, the results from the MELK-EGFP transgenic mice indicate that MELK is expressed by long-term self-renewing progenitors, consistent with the hypothesis that MELK is a self-renewal regulating protein.

MELK regulates the transition of GFAP-positive cells to a GFAP-negative, multipotent state

During the course of late embryonic and postnatal development, a population of GFAP-expressing progenitors arises in the forebrain GZs. These cells, which in the adult brain are thought to be slowly cycling, give rise to rapidly proliferative MNPs, which then are capable of generating neuronal-restricted precursors (Doetsch, 2003). Little is known about the mechanisms underlying how this progression takes place. However, because MELK mRNA was expressed by some GFAP-containing cells in the GZs and also regulates the proliferation of rapidly cycling progenitors, we hypothesized that MELK would play a role in this process.

Previous studies demonstrate that GFAP-expressing cells cultured from the neocortex—presumably the SVZ—form clonal neurospheres and produce neurons in the presence of bFGF (Laywell et al., 2000; Imura et al., 2003). Our data support the hypothesis that a subset of GFAP-positive cells express LeX and that the addition of bFGF results in the expansion of this subpopulation, which are then, in turn, multipotent, and that MELK is required for this process in vitro. It remains to be seen whether the same function is served in vivo.

Potential mechanisms of MELK function

A previous study of the human MELK orthologue pEg3 suggested that it induces phosphorylation of the cell cycle–related gene CDC25B, resulting in cell cycle arrest using ectopic expression in an osteosarcoma cell line (Davezac et al., 2002). However, our data, taken in sum, strongly indicate that MELK positively regulates the cell cycle in neural tissue. First, our functional studies demonstrated that MELK influences proliferation on cultured neural progenitors without dramatically affecting cell survival. We also found that in glioblastoma, MELK expression was highly correlated with cell cycle–promoting genes. In neural progenitor cultures, MELK expression, like many cell cycle regulatory genes, varied with phases of the cell cycle—with higher expression at S/G2/M phase than at G0/G1. Furthermore, MELK-expressing cells had different cell cycle characteristics than nonexpressing cells, a result suggestive of more rapid proliferation. Together, these data strongly support a role for MELK in the promotion of the cell cycle of rapidly proliferative progenitors. This role is unique to MELK amongst the snf1/AMPK family members.

Our expression and functional data suggest that MELK function is mediated by the proto-oncogene, B-myb. This transcription factor is known to promote G1–S transition in cell lines, and the *Drosophila* homologue myb regulates the G2–M transition (Lyon et al., 1994; Oh and Reddy, 1999; Tanaka et al., 1999). B-myb regulates the proliferation of ES cells (Iwai et al., 2001) and is required for the formation of the inner cell mass (Tanaka et al., 1999). Like MELK, B-myb is expressed in undifferentiated neurospheres, with a decline in expression during differentiation. We show that MELK knockdown downregulates B-myb expression in primary progenitors, and that B-myb knockdown also inhibits NSC proliferation. Furthermore, in vivo expression analysis also lends support to the proposed

mechanism of MELK action—through regulation of B-myb expression. There is a striking degree of overlap of MELK and B-myb mRNA expression in vivo, with all areas that express MELK mRNA also expressing B-myb mRNA.

A potential target of MELK phosphorylation and activation of B-myb is ZPR9. We also have demonstrated that ZPR9 is expressed in neurospheres and not in differentiated cells, and its expression is regulated by MELK expression. Although previous studies have suggested a direct interaction between MELK and ZPR9 as well as between ZPR9 and B-myb, our data also suggest that these factors are transcriptionally regulated by MELK. Further study will be needed to determine the precise relationship between MELK, ZPR9, and B-myb, a potential novel signaling cascade in neural progenitor proliferation.

Conclusions

In summary, MELK is a gene highly expressed in the proliferating progenitors in vivo and regulates MNP proliferation in vitro. These findings are important for the study of normal brain development, for CNS repair, and for pathological states such as brain tumors, where aberrant progenitor proliferation is implicated.

Materials and methods

Neural progenitor cultures

Neurosphere cultures were prepared as described previously (Geschwind et al., 2001). Cortical telencephalon was removed from E12 CD-1 mice, and cerebral cortex was isolated from older animals (Charles River Laboratories). In some experiments, cortices from conditional PTEN mutants were used (Groszer et al., 2001). Cells were dissociated with a fire-polished glass pipette, and resuspended at 50,000 cells/ml in DME/Ham's F12 medium (Invitrogen) supplemented with B27 (GIBCO BRL), 20 ng/ml bFGF (Peprotech), and penicillin/streptomycin (Gemini Bioproducts) and heparin (Sigma-Aldrich). Growth factors were added every 3 d. For differentiation, culture medium was replaced into Neurobasal (Invitrogen) supplemented with B27 without bFGF onto poly-L-lysine (PLL)-coated dishes, and maintained up to 5 d. For secondary sphere formation assay, the primary spheres were dissociated and plated into 96-well microwell plates in a 0.2-ml volume of growth media at 40,000 cells/ml, and the resultant sphere numbers were counted at 7 d. For rapamycin treatment, neural progenitors were incubated with 1 μM rapamycin (Sigma-Aldrich) for 2 d and stained with phospho-S6 antibody (1:300; Cell Signaling).

To assay the influence of gene knockdown or overexpression, the neurosphere culture system was modified. Neurospheres were propagated for 1 wk and then dissociated with trypsin (0.05%) followed by trituration with a fire-polished pipette. The cells were then placed in DME/Ham's F12 with 2% FBS (GIBCO BRL), and were plated onto polyornithine/fibronectin coated glass coverslips (Sun et al., 2001). After 6 h, the serum-containing medium was removed and the cells were placed back in the neurosphere growth medium without heparin and supplemented with 20 ng/ml bFGF. Transfection was then performed as described below. To assay the sphere-forming potential of the transfected cells, they were lifted off the plate with trypsin (0.05%), incubated briefly in medium containing 10% FBS to inactivate trypsin, spun, and then placed into neurobasal media supplemented with B27, bFGF, and heparin (Wachs et al., 2003). To assay the function of cells expressing EGFP driven by the MELK promoter, neurospheres at 7 d in vitro (DIV) were plated onto coverslips as above and transfected. Some cultures were then placed into neurosphere conditions to assay sphere-forming potential, whereas others were propagated and differentiated on the coated coverslips after transfection. Proliferation activity was measured by BrdU incorporation for 24 h starting at DIV3, using the Cell Proliferation ELISA BrdU (colorimetric) kit (Roche), according to manufacturer's protocol. Readout was the optical density at 492 nm. To assay cell death, living cultures were incubated for 10 min with propidium iodide (PI, 2 uM), washed twice in media, and then fixed and counterstained with Hoescht. The number of nuclei that were PI positive were counted per high power field and considered as an indicator of cell death. The morphological features of condensed (pyknotic) or fragmented nuclei were used as confirmatory measures.

GFAP-positive astrocyte-enriched cultures

Primary astrocyte cultures were prepared from P1 mouse cortices as described previously (Imura et al., 2003). In brief, as cells became confluent (12–14 DIV) they were shaken at 200 rpm overnight to remove nonadherent cells and to obtain pure astrocytes, and then were passaged onto PLL-coated coverslips for RNA collection or FGF stimulation. To determine the expression and function of MELK during the production of MNPs from GFAP-positive progenitors, the media were changed to neurosphere growth medium with bFGF. Cell proliferation and cell death were measured in the same way as for MNP.

N2A neuroblastoma cells

Mouse N2A cells (American Type Culture Collection) were cultured in DME/Ham's F12 with 10% FBS, and were passaged when confluent.

Semiquantitative RT-PCR

Total RNA was isolated from each sample using TRIzol (GIBCO BRL), and 1 μg RNA was converted to cDNA by reverse transcriptase following the manufacturer's protocol (Impron). For semiquantitative RT-PCR, the amount of cDNA was examined by RT-PCR using primers for glyceraldehyde-3-phosphate-dehydrogenase gene (GAPDH) as an internal control from 20 to 45 cycles. After correction for GAPDH signal for each set, the resultant cDNA was subjected to PCR analysis using gene-specific primers listed in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200412115/DC1). The protocol for the thermal cycler was: denaturation at 94°C for 3 min, followed by cycles of 94°C (30 s), 60°C (1 min), and 72°C (1 min), with the reaction terminated by a final 10-min incubation at 72°C. Control experiments were done either without reverse transcriptase and/or without template cDNA to ensure that the results were not due to amplification of genomic or contaminating DNA. Each reaction was visualized after 2% agarose gel electrophoresis for 30 min, and expression levels were compared between the cDNA samples on the same gel.

Quantitative RT-PCR

DNase-treated RNA samples (1 μg) were directly reverse transcribed with ImProm-II RT (Promega). Real-time PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics) according to the manufacturer's instructions. A mastermix of the following reaction components was prepared to the indicated end concentrations: 8.6 μl water, 4 μl betaine (1 M), 2.4 μl $MgCl_2$ (4 mM), 1 μl primer mix (0.5 μM), and 2 μl LightCycler (Fast Start DNA Master SYBR Green I; Roche Diagnostics). LightCycler Mastermix (18 μl) was filled in the LightCycler glass capillaries and 2 μl cDNA was added as PCR template. A typical experimental run protocol consisted of an initial denaturation program (95°C for 10 min), amplification and quantification program repeated 45 times (95°C for 15 s, 62°C for 5 s, 72°C for 15s, followed by a single fluorescence measurement). Relative quantification was determined using the LightCycler Relative Quantification Software (Roche Diagnostics), which takes the crossing points (CP) for each target transcript and divides them by the reference GAPDH CP.

In situ hybridization and immunohistochemistry

In situ hybridization with brain sections from multiple ages was performed as described previously using ^{35}S-labeled riboprobes (Kornblum et al., 1994). For double labeling using in situ hybridization and immunohistochemistry, we used the method described previously with radiolabeled riboprobes and immunohistochemistry using DAB as chromagen (Kornblum et al., 1999).

Immunocytochemistry

Immunocytochemistry of neurospheres, adherent progenitors, and neonatal astrocytes were performed as described previously (Kornblum et al., 1998; Geschwind et al., 2001) using the following antibodies: nestin (Rat401; 1:200; Developmental Studies Hybridoma Bank), LeX (CD15; 1:200; Invitrogen), TuJ1 (1:500; Berkeley Antibodies), GFAP (1:1,000; Dako Cytomation), and O4 (1:50; CHEMICON International). Primary antibodies were visualized with Alexa 568– (red), 488– (green), and 350 (blue)–conjugated secondary antibodies (Molecular Probes, Inc.). Hoechst 333342 (blue) and PI (red) were used as a fluorescent nuclear counterstain.

Sphere diameter analysis

Secondary neurospheres from E12.5 telencephalon were plated into coverslips and fixed with 4% PFA. Diameters of 30–120 randomly chosen

spheres from each condition were measured using the Microcomputer Imaging Device Program (MCID). A minimum cutoff of 40 μm was used in defining a neurosphere.

Construction of vectors

pCMV-MELK. The full-length coding region of mouse MELK was amplified by PCR using mouse embryonic neurospheres as a template, and was subcloned into pGEM-T Easy vector (Promega). After sequence verification, the MELK fragment was subcloned into pCMV-Tag vector (Stratagene) at NotI site.

PMELK-EGFP. The putative MELK promoter region was defined using PromoterScan (http://bimas.dcrt.nih.gov/molbio/proscan/). This program indicated that the 2.7 kb upstream of the starting ATG codon had multiple transcription factor binding sequences. A bacterial artificial chromosome (BAC) clone was obtained from BAC/PAC resources (Children's Hospital Oakland Research Institute, Oakland, CA). Using this BAC clone as a template, 3.5 kb and 0.7 kb upstream of the starting ATG codon of mouse MELK was amplified and subcloned into T Easy vector. After the sequence confirmation, a genomic region of MELK promoter was fused to EGFP polyA (CLONTECH Laboratories, Inc.), yielding PMELK-EGFP.

siRNA synthesis

siRNA was synthesized using the Silencer siRNA Construction Kit following the manufacturer's instructions (Ambion). Four different targeting sequences were designed from coding region of mouse MELK. Each of the four demonstrated different levels of mRNA knockdown, and one was chosen for further analysis. The sequence is listed in Table S1.

Flow cytometry and sorting

Flow cytometry and sorting of EGFP+ cells from E12- and E15-derived neural progenitors was performed with a FACSVantage (Becton Dickinson) using a purification-mode algorithm. Gating parameters were set by side and forward scatter to eliminate dead and aggregated cells. Cells transfected with a promoterless EGFP vector were used as a negative control to set the background fluorescence; false positive cells were <0.5%. For isolation of LeX+ cells (Capela and Temple, 2002), E12 progenitors were labeled with LeX antibody (Invitrogen) for 30 min and Alexa 530 was used for flow cytometry and sorting. Background signals were determined by incubation of the same set of progenitors without primary antibody.

Transient transfection

Cells were transfected using LipofectAMINE 2000 (Invitrogen) following the manufacturer's protocol. For transfection of plasmid vectors, the cells were incubated with reagents for 6 h with the primary progenitor cells, and for 24 h with N2a cells. For transfection of the double-stranded siRNA complex, serial dilutions of siRNA from 5 to 200 nM were tested to obtain specific knockdown of the gene of interest, and 100 nM was chosen as the concentration for functional study. Incubation with siRNA complex was 6 h with primary cells and 24 h with cell lines.

Image acquisition

Photomicrographs were obtained using a microscope (model IX50; Olympus) fitted with a bright- and dark-field condenser using a digital camera (model C2020; Olympus). Digital images were manipulated using Adobe Photoshop 7.0.2 in order to accurately reflect direct observation.

Online supplemental materials

Fig. S1 depicts genomic structure of human and mouse MELK and promoter characterization. Fig. S2 shows sphere formation after transfection with PCMV-EGFP. Fig. S3 shows that MELK expression is specifically altered by the expression vector and by synthesized siRNA. Fig. S4 shows that MELK function is parallel to the PTEN/Akt pathway. Table S1 lists primer sequences. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200412115/DC1.

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